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13. ABSTRACT (Maximum 200 Words)

The tumor suppressor p53 plays an important role in a variety of cancers including breast cancer. It inhibits the growth of malignant cells either by inducing G1 and G2 arrest, apoptosis or senescence. We are determining the role of p53 in human mammary epithelial cell (HMEC) senescence. We previously showed that p53 and its target gene p21 are significantly upregulated during senescence in post-selection HMECs. We also showed that cells with reduced p53 or p21 proteins have extended replicative life span. However, compared to p21 RNAi, p53 RNAi expressing cells proliferates for four more population doublings suggesting the role of additional targets of p53 in HMEC senescence. p53 is a sequence specific transcription factor, which binds to promoter region of various genes related to apoptosis and growth arrest. To identify other targets of p53, we carried out array analysis using Human TransSignal p53 Target gene array (Panomics Inc., Redwood City, CA). Briefly mRNA was prepared from early passage (proliferating) and late passage (senescent) HMECs, biotin labeled, and hybridized to p53 target gene array. Our results indicate that in general, p53 target genes are upregulated in senescent HMECs. We have identified several additional targets of p53 in senescent HMECs. These targets include p63, TSP-1, WIG1, IGF-BP3 and BCL-6. We are further confirming upregulation of these targets by RT PCR and chromatin immunorprecipitation linked PCR (ChIP) assay.

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INTRODUCTION

Cancer is a complex multistep process involving several molecular genetic changes. It is believed that the first molecular genetic change entails bypass of cellular senescence followed by the immortalization of cells (1, 2). These changes allow cells to accumulate further mutations, which results in a transformed phenotype. It is believed that breast cancer arise due to transformation of human mammary epithelial cells (HMECs), which line the ducts of mammary gland.

After completing a certain number of divisions, normal cells enter a state of irreversible growth arrest and altered function, known as cellular senescence (3), which is considered a tumor suppressive mechanism (4, 5). In somatic cells, telomerase remains repressed and telomere length keeps shortening at each round of DNA replication. Short telomeres signal cells to stop further proliferation and invoke a permanent growth arrest phenotype known as replicative senescence (6, 7). Two important tumor suppressors pRb and p53 are required for the maintenance and genesis of senescent phenotype.

The p53 protein is a typical transcription factor and contains an N-terminus transactivation, a centrally located DNA binding and a C-terminus oligomerization domains (8). Transcriptionally active p53 binds to a consensus site 5' -RRRCA/TA/TYYY-3', often present in pairs in p53 regulated genes (8). Tumor derived mutants of p53 are always defective in sequence-specific transactivation, thus attesting the importance of transcription activation function of p53. In response to various physiological stimuli, p53 undergoes post-translational modifications such as phosphorylation and acetylation, which activate p53 transcription functions (8, 9). Activation of these transcription activation functions results in either apoptosis, G1 and G2 cell cycle arrest or senescence (8-10).

When mammary tissue is explanted in an appropriate tissue culture medium, a heterogeneous cell population emerges. This heterogeneous population proliferates for 3-5 population doublings before a majority of cells undergoes senescence. Regular feeding of these cells (sometimes) give rise to a homogeneous population which is referred to post-selection HMECs, while the original heterogeneous mixture is referred to as pre-selection cells (11, 12). Senescence in pre-selection cells, which is also termed as M0 stage, appears to be due to the accumulation of p16 (13, 14), and emergence of post-selection homogeneous culture is believed to arise due to progressive methylation of p16 locus (11-14). However, post-selection cells still undergo senescence and never spontaneously immortalize.

In the previous report, we showed that p53 and p21 is significantly upregulated during senescence in post-selection HMECs but not in pre-selection HMECs. To further understand the role of p53 and its target genes in senescence of post-selection HMECs, we used RNA interference (RNAi) approach (15). Using p53 RNAi and p21 RNAi, we concluded that inactivation of p53 and/or p21 results in a significant extenstion of replicative life span but no immortalization. We also found that p53 RNAi was much more effective compared to p21 RNAi, suggesting the role of additional targets of p53 in cellular senescence in HMECs. Here, we studied role of additional p53 target genes using a p53 target gene array.

BODY:

Post-selection 76N were obtained from Dr. Vimla Band. These cells were cultured in DFCI-1 medium as described (16). Cells were serially passaged in culture until senescence. Senescence was determined using senescence associated beta-galactosidase (SA-β-gal) assay and using ^{3}H-thymdine incorporation assay (% labeled nuclei or %LN) as described (16, 17). Cells were considered early passage when >70% cells incorporated ^{3}H-thymidine and less than 5% cells were SA-β-gal positive. Conversely cells were considered senescent when SA-β-gal index was >70% and %LN were 10-15%. SA-β-gal is a widely used senescence marker used in various cell types including HMECs. To analyze p53 target genes in senescent HMECs, we obtained "Human TranSignal p53 Target Gene Array" from Panomics Inc., Redwood City, CA. The array contains 146 human p53 target genes chosen from published literature (8-10, 18). Total RNA was isolated from early passage growing and senescent HMECs, labeled with biotin-UTP using reverse transcriptase, and hybridized to individual array membranes overnight. The hybridization pattern was detected by enhanced chemiluninescence, and autoradiogram developed. The intensity of signal corresponding to various target genes by densitometry, and normalized to control spots in arrays. To knockdown p53 in HMECs, we used stable expression of p53 shRNA (short hairpin) using retrovirus (19, 20). We also used mdm2 overexpressing retrovirus in our studies (21). The retroviruses were generated as described , and HMECs expressing RNAi or gene of interest (mdm2) generated after selection as described (16, 17, 21).

RESEARCH ACCOMPLISHMENTS

1. p53 targets genes are selectively upregulated in senescent cells.

Our array data suggest that in general, p53 target genes are upregulated during senescence in HMECs (Figure 1A). Results indicated p53 regulated genes falls into four categories of genes in HMECs (Figure 1B).

A. Genes that are showed the highest upregulation in senescentcells compared to early passage proliferating cells. This category include are well charecerized p53 target genes such as *TGFα, p63, WiG1* and *TSP-1*. Other gene that are also expressed at high level compared to early passage cells were- *Jun, RB1, MAD1, TP53INP1, PRG1, HGFL1, Slac19A, 15-LO, P2RXL1, RGS14, THBS2, TOP2A, TYR AFP,AR* and *TST* (Figure 1B).
B. Genes in this category are moderately overexpressed in senescent HMECs. These genes include *IL-6. IGF-BP3,* and *BCL-6.*
C. In third category, genes showed similar level of expression in proliferating as well as senescent HMECs. These genes are *PTEN, CK8, PPM1D, MCG9* and *Killer /DR5.*
D. Finally, genes that were downregulated in senescent HMECs are *Cyclin B* and *c-myc.*

Several p53 target genes that were also present in array were not detected, this could be due to cell type specific expression of these target genes or relatively low level of expression of genes that were not detected in array analysis.

2. Generation of mdm2 overexpressing HMECs.

Mdm2 is a negative regulator of p53 that targets it for proteosome-mediated degradation. We have also generated HMECs, which stably overexpress mdm2 using retroviral LTR promoter. These cells express relatively low level of p53 similar to p53 RNAi expressing cells,

but high mdm2 (Figure 2A). mdm2 overexpressing cells also showed extension of life span and and are still proliferating whereas control vector cells underwent senescence by passage 5 (Figure 2B). We are currently characterizing these cells and comparing these cells for replicative life span and other properties with p53 and p21 RNAi expressing cells.

Early passage HMECs **Late Passage HMECs**

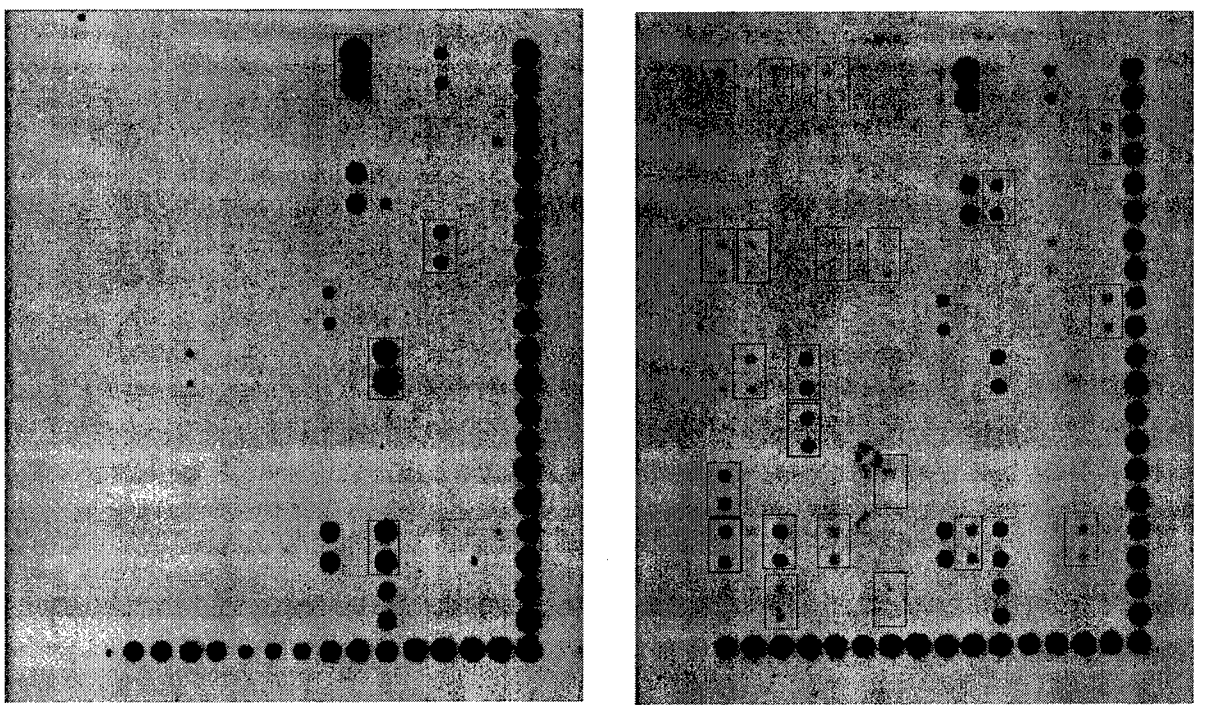

Figure 1 A : Human TranSignal™ p53 Target Gene Array. The arrays were performed with mammary epithelial cells of early and late stages. The genes on the array are spotted in duplicate. The red box indicate the high expressed genes whereas green box indicate the low expressed genes.

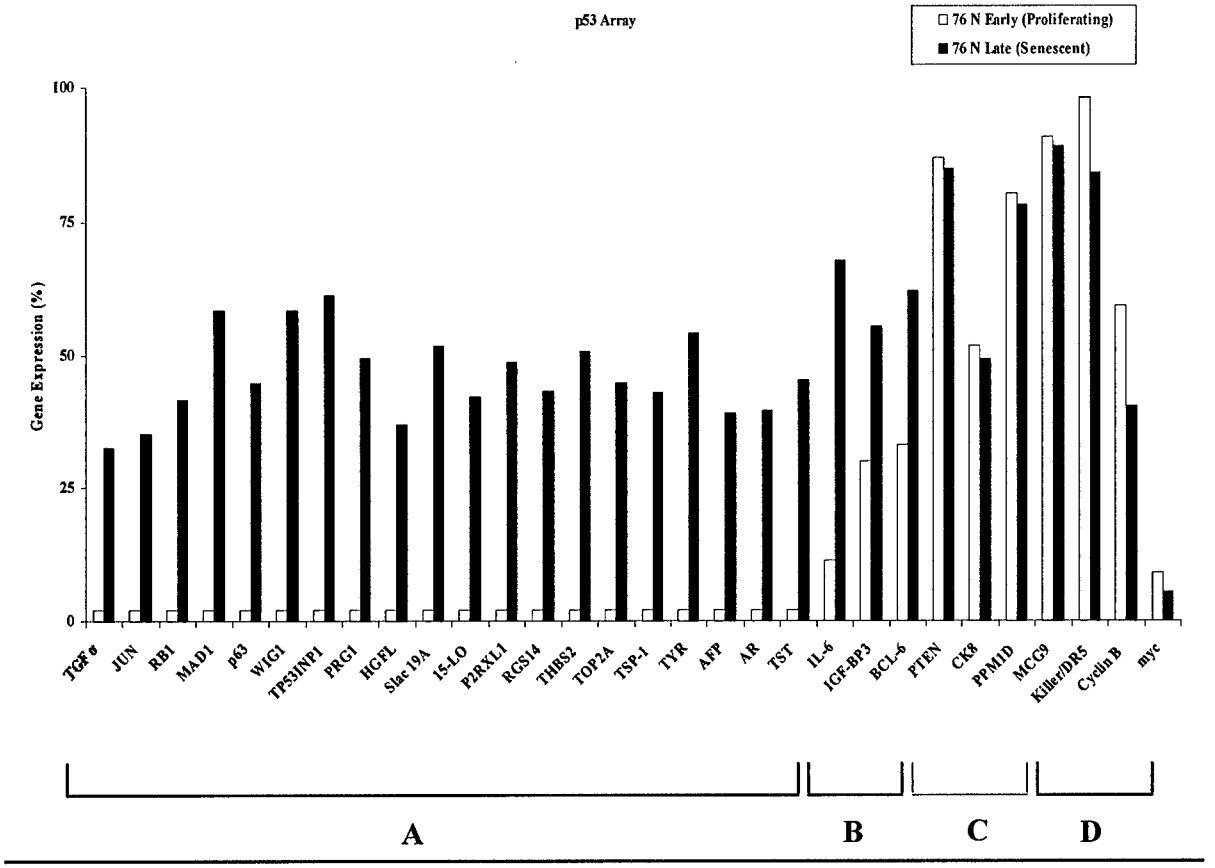

Figure 1B. Quantitative analysis of various p53 target genes present in p53 array. Genes were divided into 4 categories (A-D) as described in the text.

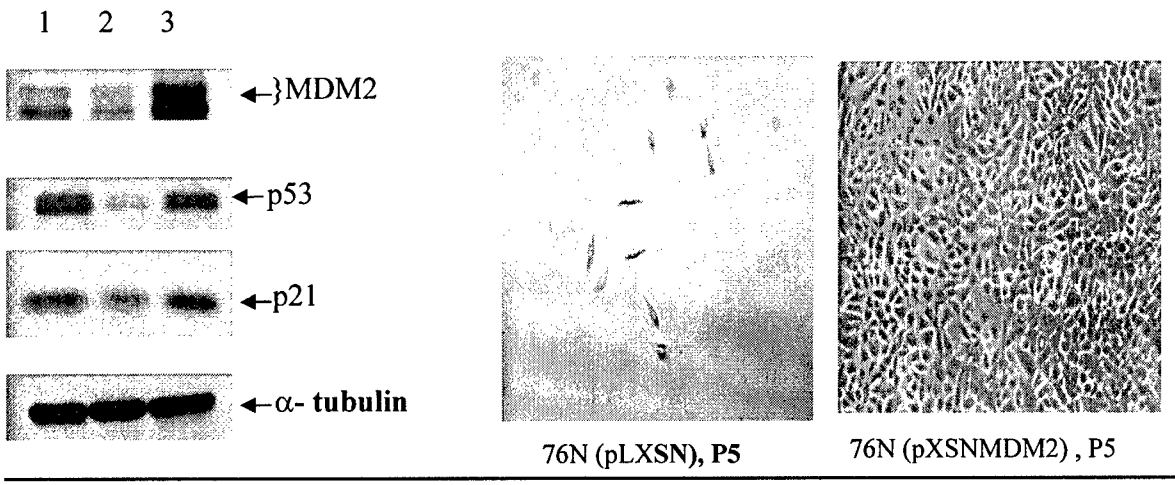

Figure 2: Overexpression of mdm2 in 76N (HMEC) cells. A- vector (lane 1), p53 RNAi expressing (lane 2) and mdm2 overexpressing (lane 3) cells were analysed by western blot analysis. B- Morphology of vector control and mdm2 overexpressing cells at passage 5 (P5).

KEY RESEARCH ACCOMPLISHMENTS:

During past three years, I have learned how to culture pre- and post-selection HMECs, and determine senescence in these cells. We have developed HMECs cell lines stable expressing p53 and p21 RNAi and mdm2. These cells are further being characterized and used to study senescence in HMECs. We will use these cells for further transformation studies and generating cell culture model of breast cancer. We have analysed p53 arrays for the expression of p53 target genes. Our data suggest role of additional p53 target genes in HMEC senescence. We are further confirming array data by RT PCR. We have also optimized p53 DNA binding and chromatin-immunoprecipitate linked PCR (ChIP) assay. The key research accomplishments during past year are following:

- p53 DNA binding activity increases with senescence in post-selection HMECs.
- p53 level and its transcription activity as determined by examining the level of its target gene p21 increases with senescence in post- but not pre-selection HMECs.
- There are no significant posttranslational changes in p53 during senescence in HMECs as determined by a limited set of antibodies.
- Stable downregulation of p53 and/or p21 using RNAi approach significantly extends replicative life span of HMECs
- Stable downregulation of p53 is more effective than p21 downregulation in extending the replicative life span of HMECs.

- Several p53 target genes are differentially expressed in senescent verses proliferating HMECs. In general p53 targets are overexpressed in senescent HMECs.

REPORTABLE OUTCOMES: None

CONCLUSIONS:

p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence. Gradual telomere shortening is thought to provoke a DNA damage checkpoint mediated by p53, which results in permanent growth arrest. Most tumor cells have lost this ability to undergo senescence and cycle even when telomere lengths critically short. In this report, we have presented evidence that p53 may play an important role in senescence of post-selection cells but not pre-selection cells.

In the first year of the grant, we proposed to study the DNA binding activity, its expression level and posttranslational modifications during senescence in HMECs. We have completed the proposed studies. However, we have not found any significant differences in posttranslational modifications using limited number of antibodies that we used.

In the second year, we started using p53 RNAi approach to study the role of p53 in senescence. We generated post-selection HMECs cells with p53 and p21 knockdown using RNAi approach. The study of replicative life span of these cells suggest that p53 plays an important role in senescence of post-selection HMECs and other target genes of p53 are possibly involved in senescence. Next year we plan to perform p53 ChIP analysis in post-selection HMECs and identify additional targets of p53 involved in HMEC senescence as previously proposed in the grant application.

In the third year we have generated mdm2 overexpressing HMECs. Cells expressing p53 and/or p21 RNAi, and mdm2 are further being charecerized in terms of senescence. In third year, we have also carried out p53 array analysis to examine the expression of p53 target genes during senescence. We have selected few p53 target genes for further study. These genes are likely to play a role in breast cancer.

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Review

Mammary epithelial cell transformation: insights from cell culture and mouse models

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Abstract

Normal human mammary epithelial cells (HMECs) have a finite life span and do not undergo spontaneous immortalization in culture. Critical to oncogenic transformation is the ability of cells to overcome the senescence checkpoints that define their replicative life span and to multiply indefinitely – a phenomenon referred to as immortalization. HMECs can be immortalized by exposing them to chemicals or radiation, or by causing them to overexpress certain cellular genes or viral oncogenes. **[AU: please check my edit of the preceding sentence.]** However, the most efficient and reproducible model of HMEC immortalization remains expression of high-risk human papillomavirus (HPV) oncogenes E6 and E7. Cell culture models have defined the role of tumor suppressor proteins (pRb and p53), inhibitors of cyclin-dependent kinases ($p16^{INK4a}$, p21, p27 and p57), $p14^{ARF}$, telomerase, and small G proteins Rap, Rho and Ras in immortalization and transformation of HMECs. These cell culture models have also provided evidence that multiple epithelial cell subtypes with distinct patterns of susceptibility to oncogenesis exist in the normal mammary tissue. Coupled with information from distinct molecular portraits of primary breast cancers, these findings suggest that various subtypes of mammary cells may be precursors of different subtypes of breast cancers. Full oncogenic transformation of HMECs in culture requires the expression of multiple gene products, such as SV40 large T and small t, hTERT (human telomerase reverse transcriptase), Raf, phosphatidylinositol 3-kinase, and Ral-GEFs (Ral guanine nucleotide exchange factors). **[AU: have I defined the terms 'hTERT' and 'Ral-GEF' correctly here (and in the abbreviations list)?]** However, when implanted into nude mice these transformed cells typically produce poorly differentiated carcinomas and not adenocarcinomas. On the other hand, transgenic mouse models using ErbB2/neu, Ras, Myc, SV40 T or polyomavirus T develop adenocarcinomas, raising the possibility that the parental normal cell subtype may determine the pathological type of breast tumors. Availability of three-dimensional and mammosphere models has led to the identification of putative stem cells, but more studies are needed to define their biologic role and potential as precursor cells for distinct breast cancers. The combined use of transformation strategies in cell culture and mouse models together with molecular definition of human breast cancer subtypes should help to elucidate the nature of breast cancer diversity and to develop individualized therapies.

Introduction

[AU: note that ref. 18 is currently cited out of sequence. Please check that it is cited correctly in the text and we shall renumber the refs if necessary.]

More than 80% of adult human cancers are carcinomas, tumors originating from malignant transformation of epithelial cells. However, much of our understanding of oncogenic transformation comes from fibroblast transformation systems. Breast cancer is the second leading cause of cancer-related deaths among women in the USA [1]. The vast majority of breast cancers are carcinomas that originate from cells lining the milk-forming ducts of the mammary gland (for review [2]). Deliberate transformation of these cells provides a practical window into human epithelial oncogenesis. Malignant transformation represents a complex multistep process in which genetic, environmental, and dietary factors together are thought to alter critical cell growth regulatory pathways resulting in uncontrolled proliferation, which is a hallmark of tumorigenesis [3,4]. Understanding the nature of these cellular pathways is of central importance in cancer biology.

The growth of normal human mammary epithelial cells (HMECs), which include luminal, myoepithelial and/or basal cells (described below), is tightly controlled. These cells grow for a finite life span and eventually senesce (for review [5-7]). Both cell culture and mouse models have provided evidence that essential initial steps in tumorigenesis involve the loss of senescence checkpoints and immortalization, which allow a

ASMA = α-smooth muscle actin; CDK = cyclin-dependent kinase; COX = cyclo-oxygenase; ER = estrogen receptor; ESA = epithelial-specific antigen; HMEC = human mammary epithelial cell; HPV = human papillomavirus; hTERT = human telomerase reverse transcriptase; PD = population doubling; Ral-GEF = Ral guanine nucleotide exchange factor; TDLU = terminal ductal–lobular unit.

cell to grow indefinitely and to go through further oncogenic steps, resulting in fully malignant behavior. In addition, cell culture model systems have identified a number of genes whose alterations are involved in HMEC immortalization and thereby have provided significant insights into the biology of early breast cancer [5,7,8]. Use of oncogene combinations has allowed researchers to create cell culture models of full HMEC transformation, thereby illuminating the process of breast cancer progression [9-11]. Additional insights have come from mouse models, using transgenic overexpression of oncogenesis-promoting genes and deletion of tumor suppressor genes, which often produce breast adenocarcinomas that closely resemble human breast cancers.

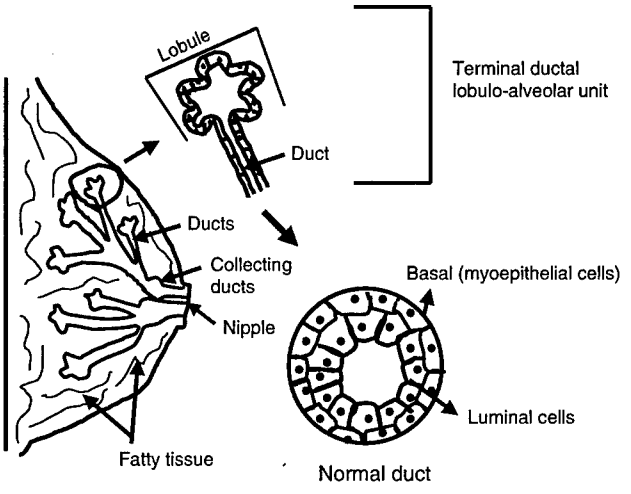
Studies using cell culture transformation models have pointed to the existence of HMEC subtypes with distinct susceptibilities to oncogenesis by different oncogenes [5,8]. Remarkably, direct cDNA microarray profiling of human breast cancers has led to similar insights, identifying multiple subtypes of human breast cancer with distinct outcomes; phenotypic and genotypic characteristics of these breast cancer subtypes point to their possible origin from specific subtypes of HMECs, such as basal or luminal cells [12]. Finally, cell culture and mouse model systems have begun to identify mammary stem cells that may provide progenitors for oncogenic transformation [13] and have led to an appreciation of the microenvironment for oncogenesis [14,15].

Thus, studies conducted over the past several years have established the importance of HMEC transformation models to our understanding of the pathways that control normal mammary cell growth, development, and oncogenesis. However, many challenges remain, including the identification of mammary cell subtypes or oncogenic strategies that result in cancers that resemble naturally occurring human breast cancers, and translation of new research to devise more specific diagnostic and treatment strategies for different subtypes of breast cancer.

Mammary gland and various epithelial cell subtypes

The mammary gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structures, termed the terminal ductal–lobular units (TDLUs), together with interlobular fat and fibrous tissue [16,17]. **[AU: please check my edit of the preceding sentence.]** Most breast cancers arise in the TDLU (Fig. 1). Unlike other epithelial cancers, such as that of colon, different stages of breast cancer are not clearly defined. However, it is clear that benign stages (such as typical and atypical hyperplasia), noninvasive cancers (such as carcinoma *in situ* – ductal or lobular), and invasive cancers (such as invasive ductal or lobular carcinomas) do exist. Additionally, multiple types of *in situ* carcinomas, such as solid, cribiform, papillary and comedo types, have been reported and it is possible that these represent tumors originating from different epithelial subtype [16,17].

Figure 1



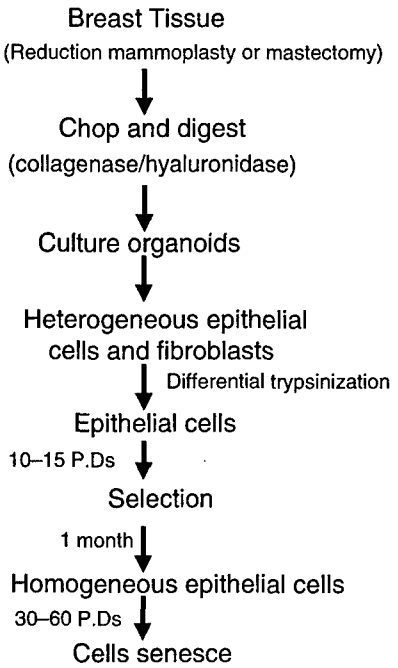
Structure of the mammary gland. Terminal ductal lobulo–alveolar unit (TDLU), composed of ductal cells, is the unit thought to be the origin of most breast cancer. **[AU: in the text 'TDLU' is defined as 'terminal ductal–lobular unit'. Should the definition be changed here?]** The stroma is composed of fatty tissue (adipocytes) and fibroblasts. Also shown are the two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells. A putative progenitor/stem cell is also indicated.

Histological examination of TDLU reveals two major types of cells: inner secretory luminal cells and outer contractile myoepithelial cells (Fig. 1). In addition to luminal and myoepithelial cells, there is emerging evidence that basal cells (presumed to be the progenitor for myoepithelial cells) and stem cells exist in the TDLU [17]. Until recently it was believed that the vast majority of breast carcinomas arise from luminal epithelial cells [2]. This was based on the keratin expression and other phenotypic markers of cultured tumor cell lines, mostly derived from metastatic lesions [2]. Unfortunately, the great majority of primary breast tumors have proved difficult to establish in cultures, either on plastic or as three-dimensional cultures [5-7,19-21]. However, recent molecular profiling studies clearly show the existence of multiple subtypes of breast cancers probably originating from luminal, basal, and possibly stem cell compartments [12] (described below in detail).

Culturing of various epithelial cell subtypes

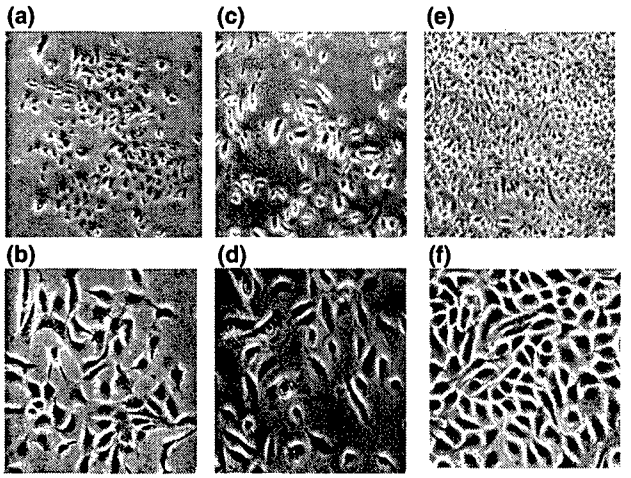
For more than two decades, various investigators have attempted to develop cell culture models that lead to isolation of breast cancer cells resembling those found in human breast cancers. In order to establish such models, it was essential to culture normal HMECs. In 1980s, work from several laboratories showed that normal HMECs could be cultured in cell culture [22,23] (for review [2,5,7]).

Figure 2



Establishment of mammary epithelial cells from reduction mammoplasty/mastectomy specimens. The tissue is chopped, digested with collagenase and hyaluronidase, and plated in medium as organoids. Over a week or so, multiple types of epithelial cells and fibroblasts emerge; fibroblasts are removed by differential trypsinization (fibroblasts are loosely attached), remaining epithelial cells grow for 10–15 population doublings (PDs) followed by senescence of the majority of cells. Occasionally, an homogenous population of cells emerges that continue to proliferate for an additional 30–60 PDs, and eventually these cells also senesce (this step is referred to as agonescence).

Figure 3



Morphological heterogeneity of cells before and after selection. **(a–d)** Two views of mammary epithelial preselection cells (original magnifications: panels a and c, 40×; panels b and d, 100×). Cells shown in panel a grow as compact clusters and are relatively uniform, whereas cells in panel b grow more dispersed and exhibit different types of cells (small and large). **(e,f)** Views of postselection human mammary epithelial cells with relatively uniform morphology (original magnifications: panel e, 40×; panel f, 100×). **[AU: please check my edit of the figure legend.]**

In our laboratory we defined a medium, termed DFCI-1, that helped us to establish and culture normal and some primary breast cancers under identical conditions [20]. However, in general the difficulty in establishing primary tumor cells in cell culture has persisted. Notably, early cultures derived from reduction mammoplasty or mastectomy specimens exhibit considerable heterogeneity (with multiple cell types – luminal, stem cells, basal and myoepithelial cells) and grow for three to four passages or about 15–20 population doublings (PDs), and then senesce (Figs 2 and 3) [5-7]. The senescence in these cells is also termed as M0 stage [24]. However, in some cases (not always) an occasional homogenous cell population emerges that continue to grow further for 30-60 PDs (Figs 2 and 3) [5-7] before senescence occurs (also called agonescence, described below) [25]. This process of emergence of cells that are able to proliferate for extended periods is also known as self-selection; before selection the cells are termed preselection cells, whereas those that emerge after selection are called postselection cells. The keratin profile of preselection cells (K-5, K-6, K-7, K-14, K-17, K-18 and K-19 positive) [8,19,26] suggests the existence of both luminal and basal (myoepithelial) cells. However, postselection cells generally exhibit a loss of expression of K-19 but retain the expression of all other keratins [8,18,25]. These cells also express α-smooth muscle actin (ASMA), suggesting that these may be of myoepithelial origin. Further development of cell sorting techniques and chemically defined media have helped in culturing of luminal and progenitor epithelial cells [14,27] (described below in detail).

It has also been reported that postselection cells lose the expression of $p16^{INK4a}$, a cyclin-dependent kinase (CDK) inhibitor [24,25], and gain expression of cyclo-oxygenase (COX)-2, a gene that is thought to be involved in tumorigenesis [28]. As both of these genes are implicated in oncogenesis, it is conceivable that loss of p16 or gain of COX-2 expression may make these cells more susceptible to transformation, although it is unclear whether the loss of p16 and gain of COX-2 occur *de novo* during self-selection or represent selection of a minor population of cells with pre-existing high COX-2 and low p16 expression. Notably, p16-negative and COX-2-positive cells could be detected using immunohistochemistry in normal mammary tissue [29].

Immortalization of various HMEC subtypes in culture

As alluded to above, normal mammoplasty-derived HMECs exhibit a limited life span, which is followed by replicative

senescence. Replicative senescence acts as a strong tumor suppressor mechanism and prevents spontaneous immortalization of human cells [30-33]. A major determinant of replicative senescence is the enzyme telomerase, which maintains the length of telomere ends [30,31]. Most somatic cells express little or no telomerase, resulting in telomere shortening with successive cell divisions, which eventually elicits a senescence checkpoint [30-32]. A senescence-like phenotype can also be induced by a variety of nontelomeric signals such as DNA-damaging agents, adverse cell culture conditions, and overexpression of certain oncogenes [30,32]. The tumor suppressor protein p53 and its target gene product p21, and $p16^{INK4a}$ play a crucial role in senescence induced by telomeric as well as nontelomeric signals [30-33]. Much of our knowledge about senescence comes from studies conducted in human fibroblasts [30-34]. Only recently have we begun to elucidate the mechanisms of senescence in epithelial cells, in particular in HMECs [25].

The senescence associated with the 'selection' phase in HMEC cultures is accompanied by classic features of senescence, such as flat morphology, presence of vacuoles, and positive staining for senescence-associated β-galactosidase (SA-β-gal), a marker of senescence [34]. The block in cell proliferation at this stage is dependent on the pRb/p16 pathway [24,35], because the human papillomavirus (HPV) oncogene E7, which binds and inactivates pRb, can overcome the M0/selection stage [36]. Similarly, a constitutively active p16-insensitive CDK4 mutant can overcome the M0 stage [37]. Thus, senescence of preselection cells appears to be telomere independent. At the end of their replicative life span, postselection HMECs exhibit senescence as well as cell death with a high level of genomic instability. This phenomenon is termed as agonescence, as opposed to replicative senescence [25]. Most importantly, unlike rodent cells, human HMECs derived from reduction mammoplasties or from milk do not exhibit spontaneous immortalization and thus provide suitable models of human cell transformation. Immortalization of HMECs in culture is characterized by their continuous growth beyond the agonescence checkpoint. It is thought that immortalization is an early step in human cancer, and continued proliferation of immortal cells allows the accumulation of additional genetic changes that promote malignant and metastatic behavior.

Stampfer and Bartley [38] presented initial evidence that HMECs could be immortalized in cell culture using benzo(a)-pyrene; however, the immortalization was a rare event in this case. Similar to carcinogen-induced immortalization, we found that γ-radiation induced the transformation of HMECs relatively infrequently [5,8,39]. In general, most viral oncogenes (including SV40 T antigen, adenovirus E1A and E1B, polyoma T antigen) have not proven very efficient as immortalizing genes for human cells [40]. While the introduction of the SV40 T antigen into breast tumor tissue-derived epithelial cells gave rise to immortal cell lines, SV40-transfected cells go through a long crisis period, and emergence of immortal cells is rare [19]. Over the past several years, our studies have defined a system to immortalize human HMECs efficiently and reproducibly, using the urogenital carcinoma-associated HPV oncogenes E6 and E7 [5,8,36].

Comparison of early (preselection) and late-passage (post-selection) cultures revealed that different HMEC subtypes exhibit a remarkably distinct susceptibility to E6 or E7, or their combination [8]. One HMEC subtype was exclusively immortalized by E6 but not by E7; such cells predominated the late-passage cultures but were rare at early passages. Surprisingly, a second cell type, present only in early passages of tissue-derived cultures, showed extension of life span and infrequent immortalization by E7 alone. Finally, E6 and E7 together were required to immortalize fully a large proportion of preselection HMECs [8].

Human milk is an easily available source of relatively pure HMECs that are thought to be differentiated luminal cells [2,19]. However, these cells can be cultured for only a limited number of passages (typically two to three passages, or five to nine PDs), which has precluded their detailed biochemical study [2,18]. Most of the work on milk cells has been carried out in Taylor-Papadimitriou's laboratory and has demonstrated that these cells can be immortalized by SV40 T antigen [41]. Interestingly, neither E6 nor E7 alone could induce the immortalization of milk-derived HMECs, whereas a combination of E6 and E7 was effective [8].

The reproducibility and relatively high efficiency with which E6 (in postselection HMECs) or E6 and E7 combined can induce immortalization of human HMECs have therefore yielded a practical approach to elucidate the biochemical mechanisms of HMEC immortalization. In recent years, using the HPV16 E6 as bait in yeast two hybrid analyses of a HMEC cDNA library, we identified several known as well as novel targets of the E6 oncogene that represent potential mediators of E6-mediated HMEC immortalization [5]. **[AU: the text 'using the HPV16 E6 as bait in yeast two hybrid analyses of a HMEC cDNA library' is a little difficult to follow – can you suggest a rephrase?]** These include ADA3 (alteration/deficiency in activation 3), a novel coactivator of p53 and steroid receptors (estrogen receptor [ER] and retinoic acid receptor) [42-44]; E6 targeted protein 1 (E6TP1), a novel GTPase activating Rap small G protein; and protein kinase N (PKN), an effector for Rho small G protein [5]. We recently found that MamL1, a human homolog of the *Drosophila* mastermind gene and a known coactivator for Notch [45], also interacts with E6 (Bhat, Band, unpublished data). **[AU: please specify the initials for Bhat and Band.]** These studies have implicated the p53, Notch, ER, Rho, and Rap signaling pathways in early transformation of human HMECs. Consistent with these analyses, we have shown that expression of mutant p53 [46] or activated Rho

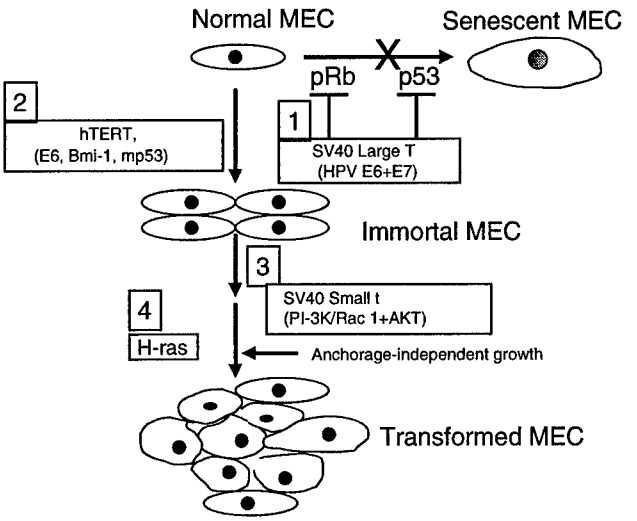
(Zhao, Band, unpublished data) induces immortalization of HMECs. **[AU: please specify the initials for Zhao and Band.]** Furthermore, several studies support a role for p53 mutations as an early event in breast cancer [47]. Taken together, these studies demonstrate that E6 is the most efficient immortalizing gene for postselection HMECs and that E6 immortalizes the HMECs by concurrently altering multiple biochemical pathways. Future studies will need to address the precise role played by these novel oncogene targets in early breast cancer.

In addition to viral oncogenes, alterations in the expression of cellular genes can also help to overcome senescence and promote HMEC immortalization. Among the cellular genes, we recently reported that Bmi-1, a member of the polycomb group of transcriptional repressors, could immortalize postselection HMECs [48]. Although the detailed mechanism of immortalization induced by Bmi-1 remains to be explored, Bmi-1 does not appear to immortalize these cells by down-regulating the INK4a/ARF locus. Interestingly, recent studies have implicated Bmi-1 in stem cell function and renewal [49,50], suggesting that Bmi-1 could function as a potential breast cancer stem cell marker [50]. Another study showed that ZNF217, a zinc finger protein that is overexpressed in breast cancers, can promote immortalization of postselection HMECs [51]. Furthermore, introduction of hTERT, the catalytic component of telomerase, also induces immortalization of postselection cells [5]. Interestingly, induction of telomerase has been documented early after E6 was introduced into HMECs [52], although the cause and effect relationship between telomerase induction and E6-induced immortalization continues to be debated. Recently, the E6 and E6–AP binding protein NFX-91 was implicated in E6-mediated induction of telomerase [53].

Cell culture models of full transformation of HMECs

The ability of researchers to establish normal HMECs and to induce their reproducible immortalization has provided momentum for further efforts to define the nature of biochemical alterations that can lead to full oncogenic transformation. As we and others have demonstrated, HMECs immortalized by most currently known procedures (such as E6 or E6 plus E7, mutant p53, Bmi-1 and human telomerase reverse transcriptase [hTERT]) are preneoplastic and do not grow in an anchorage-independent manner or produce tumors when implanted in immune-deficient mice [5,8]. Weinberg and colleagues [9] recently established a multistep model of full HMEC transformation in cell culture by serial introduction of SV40 large T and small t, hTERT, and activated Ras (Fig. 4). It was shown that introduction of the SV40 large T, which binds and inactivates p53 and pRb, abolished senescence, whereas hTERT was needed to promote immortalization [9]. Notably, these studies showed an essential role for the SV40 small t, which inhibits protein phosphate 2A [54]. HMECs transformed by this method

Figure 4



Current consensus: normal HMECs can be fully transformed in definable serial steps. The first step, bypass of senescence, is achieved by inactivation of p53 and pRb by SV40 large T, human papillomavirus (HPV) E6 and E7, or by inhibition of p53 and pRb expression by the RNAi approach (or expression of dominant-negative mutants in the case of p53). The second step, immortalization, is achieved through the expression of hTERT. Alternatively, expression of HPV E6 or overexpression of Bmi-1, mutant p53, or ZNF217 can be used to induce immortalization of HMECs. The third step, anchorage-independent growth, can be achieved by SV40 small t mediated modulation of PI3K and/or other signaling pathways or by overexpression of activated Rac1 and AKT. The fourth step, full transformation, requires the introduction of activated H-ras, which can be substituted by Raf and Ral-GEFs. Although the current model systems have utilized the serial schemes depicted, other combinations and/or schemes of oncogene introduction are likely also to be effective. Adapted from Elenbaas [9], Zhao [10], and Rangarajan [11] and coworkers. HMEC, human mammary epithelial cell; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; PI3K, phosphatidylinositol 3-kinase; Ral-GEF, Ral guanine nucleotide exchange factor; RNAi, RNA interference. **[AU: please check my edit of the figure legend.]**

exhibited anchorage independence and produced poorly differentiated carcinoma (but not adenocarcinoma) when implanted in nude mice [9]. Further dissection of the role of small t revealed the importance of the downstream targets of phosphatidylinositol 3-kinase, Akt1 and Rac1, and direct activation of these pathways could fully substitute for small t in the transformation assays [10]. A recent refinement of the transformation in cell culture scheme suggests that perturbation of p53, pRb, protein phosphate 2A, telomerase, Raf, and Ral guanine nucleotide exchange factor (Ral-GEF) pathways are required for the full tumorigenic conversion of normal human cells [11]. The requirement in terms of modulating Raf and Ral-GEF pathways is cell type specific; HMECs require activation of Raf, phosphatidylinositol 3-kinase and Ral-GEFs, whereas human fibroblasts require the activation of Raf and Ral-GEFs [11]. Thus, serial use of viral

and/or cellular genes is beginning to unravel the various combinations of genetic lesions that can convert a completely normal mammary epithelial cell into a fully tumorigenic one.

Although these studies have thus far relied on the use of known oncogenes, future studies using the cell culture transformation models with gene libraries should help identify novel cellular genes that participate at various steps of breast cancer progression. Vast majority of human breast cancers are adenocarcinomas, and only a small portion of breast cancers are poorly differentiated carcinomas. Hence, it appears that HMEC transformation in culture system is not optimal because the tumors produced by these HMECs have usually been poorly differentiated carcinomas rather than adenocarcinomas. Breast cancer is associated with overexpression of various cellular proto-oncogenes such as ErbB2, epidermal growth factor receptor, Src family kinases, Bmi-1, cyclin D_1, cyclin E, CDK4, and other potential growth regulators. Use of these oncogenes in the multigene model described above and the use of other HMEC subtypes (such as luminal cells, potential stem cells, or those derived from milk) as a starting population may help to achieve full transformation of HMECs that develop into adenocarcinomas in a mouse model. **[AU: is the text 'full transformation of HMECs that develop into adenocarcinomas in a mouse model' correct as edited?]** Thus, future studies must focus on developing models that will lead to breast tumors that faithfully reproduce the pathological characteristics of human breast cancers.

Transgenic mouse models of breast cancers

Mouse models of breast cancers have provided a wealth of knowledge about the molecular pathways involved in breast cancers. Initial studies in these models used carcinogens to induce breast carcinomas [55]. Later studies targeted a wide variety of genes expressed under either the MMTV (mouse mammary tumor virus) or the WAP (whey acidic protein) promoter to target genes to the mammary gland. Importantly, such studies invariably produced breast adenocarcinomas in mice that resembled human breast cancers. These include viral proteins, such as SV40 large T, polyoma virus T antigen [56-58], or cellular proteins such as c-Myc, ErbB2/neu, cyclin D_1, cyclin E, ERs, mutant p53, c-Ha-ras, and Wnt-1 [59-63]. Recent studies have focused on mouse models with either a global or a mammary-specific knockout of specific genes to examine the function of obvious players, such as cell cycle related proteins and tumor suppressors, either by themselves or after these deficiencies were combined with transgenic neu or other oncogenes. For example, cyclin D_1-deficient mice are resistant to mammary carcinomas induced by c-neu/ErbB2 and Ha-ras but not to those induced by c-Myc or Wnt-1 [63]. These findings define a pivotal role for cyclin D_1 in selective mammary cancers in a mouse model and imply a functional role for cyclin D_1 overexpression in a subset of human breast cancers. In another study, Cre-mediated deletion of exons 3 and 4 of the mouse *Brca2* gene in mice with a loxP-modified and null *Brca2* allele resulted in high incidence of breast adenocarcinomas [64]. Similarly, the telomere attrition in aging telomerase-deficient and p53-mutant mice promoted the development of breast adenocarcinomas [65]. Another study showed that loss of Stat5a delays mammary cancer progression in a WAP-TAg transgenic mouse model [66].

Collectively, these models have defined a role for p53, Rb, BRCA1/2, cyclins, CDKs, ErbB2, c-Myc, Wnt-1, ER, and progesterone receptor in mammary cell growth and development of breast cancers. Finally, these different oncogenes and the pathways in which they work seem to target different progenitors or cell types in mammary gland to develop mammary tumors [67]. For example, the Wnt signaling pathway targets both luminal and myoepithelial cells, whereas Neu, H-Ras, and polyoma middle T antigen target only luminal epithelial cells [67]. The take-home lesson here is that the majority of these mouse models result in tumors that resemble human breast adenocarcinomas pathologically. The lack of development of adenocarcinomas from cells transformed in culture models may thus reflect the cell type that was used as the starting normal cell, rather than any peculiarity associated with the use of mouse as a host. **[AU: please check my edit of the preceding sentence.]**

Molecular classification of breast cancers: cues from cell culture studies

A vast body of clinical literature indicates that breast tumors exhibit diverse phenotypes as judged by their distinct clinical course, pathological features, and responsiveness to various therapies. However, it has not been clear whether this diversity reflects cancers arising from distinct subtypes of HMECs. Consistent with such a possibility, several years ago we reported the presence of different subtypes of cells in reduction mammoplasty specimens and in milk that exhibited differential susceptibility to viral oncogenes [5,8]. Direct evidence for the conclusions derived from these cell culture studies was provided by recent work utilizing gene expression patterns in primary human breast cancers, using cDNA microarrays. These studies identified distinct gene expression profiles or molecular portraits based on which breast tumors could be subclassified into groups that appear to reflect the original cellular subtypes found in the mammary gland [12]. Five categories of breast cancers were described [12]: a basal epithelial-like group, an ErbB2-overexpressing group, a normal breast epithelial-like group, luminal epithelial cell type A, and luminal epithelial cell type B. A slightly different classification was proposed by Sotiriou and coworkers [68]. The breast tumors were first divided into ER-positive and ER-negative categories. The ER-negative tumors were further subgrouped into basal-like 1, basal-like 2, and ErbB2/neu tumors, whereas ER-positive tumors were subdivided into luminal-like 1, luminal-like 2, and luminal-like 3 subtypes. Sotiriou and coworkers also re-examined data from the study by Sorlie and coworkers [12] and suggested that

luminal-like breast cancer could be classified as luminal A, B, and C subtypes corresponding to luminal-like 1, luminal-like 2, and luminal-like 3 subtypes.

Interestingly, survival analyses conducted in a subcohort of patients with locally advanced breast cancer uniformly treated in a prospective study showed significantly different outcomes for the patients belonging to the various groups, with the basal-like subtype correlating with worst outcome, followed by ErbB2 overexpressing, normal cell type and luminal cell type groups [12,68]. Interestingly, a significant difference in outcome for the two ER-positive groups was also noticed [68]. These studies strongly support the idea that many of the breast tumor subtypes may represent malignancies of biologically distinct cell types producing distinct disease entities that may require different treatment strategies. Importantly, these analyses provide a strong rationale for further definition of various mammary epithelial subtypes and expansion of immortalization and full transformation strategies to derive models that may faithfully reproduce the histological and molecular diversity encountered in human breast cancers.

Do breast cancers arise from stem cells?

Stem cells have enormous replicative potential and capacity for self-renewal, and give rise to different lineages of cells. Although still a controversial notion, many cancers are thought to originate from cancer stem cells [69]. This idea has also attracted a great interest in the field of breast cancer research, and investigators have begun to examine whether there are mammary stem cells [13,17,27,70-73]. The cellular milieu of the mammary gland undergoes significant changes during pregnancy, lactation, and involution. These include bursts of proliferation of existing cells during pregnancy, continued differentiation during lactation, and apoptosis during involution at the end of the cycle. This cyclical behavior predicts the presence of a stem cell-like population in the mammary gland, which would meet the demand of a pregnancy cycle. The existence of adult mammary epithelial stem cells has therefore been proposed. Direct evidence for the existence of such cells has come from clear fat-pad transplantation, retroviral tagging, and X-chromosome inactivation studies in rodent model [13,16,17,70-73].

Recently, using various putative stem cell and cell surface markers, such as sialomucin (Muc), epithelial-specific antigen (ESA), various cytokeratins, ASMA, and CALLA or CD10, attempts have been made to identify the mouse and human mammary epithelial stem cells [13,27,70-73]. Using immunomagnetic cell sorting based on surface antigen markers (Muc and ESA) and subsequent immortalization with E6 and E7, Gudjonsson and coworkers [27] separated $Muc^-/ESA^+/K\text{-}19^+$ cells that were able both to self-renew and to give rise to Muc^-/ESA^+ epithelial cells and $ASMA^+$ myoepithelial cells, thus exhibiting characteristic of breast stem cells. Dontu and coworkers [13] isolated undifferentiated mammospheres from single cell suspensions of HMECs obtained by mechanical and enzymatic dissociations. Primary mammospheres can be further passaged to generate secondary mammospheres. Primary as well as secondary mammospheres were highly enriched in early progenitor or stem cells capable of differentiating along multiple lineages and of self-renewal. Immunostaining of these mammospheres showed the presence of CD10, α_6 integrin and K-5 on early progenitors, and ESA and K-14 on late progenitor cells [13]. However, Muc1, K-18, and ASMA were not expressed in cells present in mammospheres [13]. Detailed expression profiling of mammospheres suggests the presence of additional markers that are upregulated in mammospheres such as stem cell growth factor, hepatocyte growth factor antagonist, stem cell growth factor B and apolipoprotein E. **[AU: I replaced 'HGF' with 'hepatocyte growth factor' – correct?]** Some markers are exclusively expressed in mammospheres such as FZD2 (frizzled homolog 2), glypican 4, interleukin-6, CXCR4 (CXC chemokine receptor), and FGFR1 (fibroblast growth factor receptor 1). Several genes that are expressed in mammospheres are also expressed in similar structures derived from other cell types (such as neurospheres formed by neural stem cells) [13].

Thus, culture of human HMECs in mammospheres may provide a tool with which to isolate and study mammary epithelial stem cells and their oncogenic susceptibilities. Based on the above and other related studies [13,17,27], the candidate mammary stem cells appear to be ESA^+, $Muc1^-$, α_6 $integrin^+$, and $CD10^+$, and the mammary stem cell niche appears to be at the suprabasal location within the luminal cell layer. Further work by other laboratories and adoption of the schemes employed by Gudjonsson [27] and Dontu [13] and their groups should help in determining the general feasibility of these novel approaches.

Apart from normal mammary stem cells, the possible existence of a breast cancer stem cell has been reported in the literature [74,75]. In a NOD/SCID xenotransplants model, Al-Hajj and coworkers [75] used four cell surface markers, CD44, CD24, ESA and B38.1 (a Breast/ovarian cancer specific marker), and lineage markers to sort different populations of breast cells from breast tumor tissues. All mice injected with $Lin^-/CD44^+/B38.1^+/CD24^{-/low}$ generated tumors, whereas none of the mice injected with $CD44^-/B38.1^-$ cells developed tumors. $Lin^-/CD44^+/B38.1^+$ fractions were further subdivided based on ESA expression. When used in numbers as low as 200, $Lin^-/ESA^+/CD44^+/CD24^{-/low}$ cells in xenotransplants generated tumors that were similar to initial tumors in term of phenotypic heterogeneity [75]. The presence of such a population in breast tumor tissue, which is able to self-renew and differentiate, supports the stem-cell model of breast tumorigenesis.

Conclusion

Our ability to culture and immortalize normal HMECs has provided a wealth of knowledge about the behavior of mammary cells and the genes involved in normal cell growth and oncogenesis. Characterization of these cells has provided novel markers that may permit early diagnosis and prognostication of breast cancers, and has yielded knowledge about potential precursor cells for breast cancers. **[AU: please check my edit of the preceding sentence.]** Transformation analyses in cell culture models have also proven important to our understanding of the multistep nature of breast cancer. Transgenic mouse models have identified the roles played by various tumor suppressors, cell cycle proteins, and other proto-oncogenes in breast cancers. Recent studies using three-dimensional models have proven useful to our understanding of the normal and tumor mammary stem cells and the relationship of microenvironment to epithelial cell growth. Finally, using gene profiling, we have begun to appreciate that breast cancers do not originate only from luminal cells but also from basal and myoepithelial cells, and that there are subtypes of breast cancers that possibly originate from distinct normal precursors that have distinct clinical outcomes and may require different treatment strategies.

However, a number of critical questions remain. What are breast stem cells and what is their role in breast cancer? Are myoepithelial cells and basal cells similar or distinct? Why can we not culture most of the primary breast cancers? How can we develop transformed breast cells in culture that would give rise to breast tumors that resemble human breast cancer – adenocarcinomas as opposed to poorly differentiated carcinomas? How do different subtypes of breast cancer originate?

In conclusion, experimental immortalization and transformation models have led to substantial progress in our understanding of the biology of breast cancer. Future studies in these model systems should go a long way toward elucidating the nature of breast cancer heterogeneity and thus facilitate the development of more individualized therapies for breast cancer patients.

Competing interests

The author(s) declare that they have no competing interests.

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